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DETERMINATION OF MINIMAL INFECTIOUS DOSE OF AN ENTEROVIRUS IN DRINKING WATER

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16. ABSTRACT

The goals of this project were to determine the minimal infectious dose and medical significance of an enteric virus ingested in drinking water. The study was conducted under double-blind, placebo-controlled, random-selection conditions. A total of 149 susceptible (antibody-free), healthy, young adult males ingested varying doses of ECHO-12 virus (10-10,000 pfu) or placebo seeded into 100 ml of non-chlorinated water and were followed for evidence of illness, viral shedding, and antibody response. There was no illness in any of the subjects and no evidence for infection among the placebo subjects. Infection in exposed subjects was primarily demonstrated by fecal shedding of virus. Only four subjects experienced a significant seroconversion without evidence of fecal shedding. Similarly, pharyngeal shedding of virus was found in only five subjects without evidence of concomitant fecal shedding. Infection occurred in 30% of the subjects given 10 pfu, the lowest dose administered, and 100% (12/12) were infected at a dose of 300 pfu as measured in LLC-MK_2 cells. Intestinal shedding persisted for as long as 4 weeks in some subjects. Statistically, it was inferred from probit analysis that ingestion of 1 to 2 pfu of ECHO-12 virus would infect 1% of the population.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and nonionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

This report presents the findings of an extensive virus infectious dose study conducted with human volunteers. With data of this type, the potential health hazard of low levels of virus in drinking water will be better understood thereby leading to appropriate corrective action.

F. Gordon Hueter, Ph.D
Director
Health Effects Research Laboratory

ABSTRACT

The goals of this project were to determine the minimal infectious dose and medical significance of an enteric virus ingested in drinking water. The study was conducted under double-blind, placebo-controlled, random-selection conditions. A total of 149 susceptible (antibody-free), healthy, young adult males ingested varying doses of ECHO-12 virus (10-10,000 pfu) or placebo seeded into 100 ml of non-chlorinated water and were followed for evidence of illness, viral shedding, and antibody response. There was no illness in any of the subjects and no evidence for infection among the placebo subjects. Infection in exposed subjects was primarily demonstrated by fecal shedding of virus. Only four subjects experienced a significant seroconversion without evidence of fecal shedding. Similarly, pharyngeal shedding of virus was found in only five subjects without evidence of concomitant fecal shedding. Infection occurred in 30% of the subjects given 10 pfu, the lowest dose administered, and 100% (12/12) were infected at a dose of 300 pfu as measured in LLC-MK_2 cells. Intestinal shedding persisted for as long as 4 weeks in some subjects. Statistically, it was inferred from probit analysis that ingestion of 1 to 2 pfu of ECHO-12 virus would infect 1% of the population.

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LIST OF ABBREVIATIONS

HI	-- hemagglutination-inhibition
LLC-MK_2	-- continuous rhesus monkey kidney tissue culture cell line
pfu	-- plaque-forming units
PBS	-- phosphate-buffered saline
CPE	-- cytopathic effect
$TCID_{50}$	-- tissue culture infectious dose 50
MEM	-- minimum essential medium
FCS	-- fetal calf serum
CBC	-- complete blood count
SGOT	-- serum glutamic-oxaloacetic transaminase
SGPT	-- serum glutamic-pyruvic transaminase
BUN	-- blood urea nitrogen

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SECTION 1

INTRODUCTION

Waterborne outbreaks of viral disease have been documented in the past and continue to occur (1-3). There have been large outbreaks of waterborne viral disease which have not been properly investigated and/or defy precise epidemiological definition and investigation (1-2). In the past several decades, significant efforts have been directed towards the development of reliable, sensitive techniques for the detection of viruses in water supplies (4). Indeed, small amounts of viruses have been found in waters treated in various manners and degrees (5). Viruses which are known or highly suspected to contaminate treated waters are hepatitis A virus (now tentatively classified as an enterovirus), enteroviruses, adenoviruses, and the group of nonbacterial gastroenteritis viruses (rotaviruses, Norwalk agent) (2,6). There is evidence that enteroviruses occasionally survive water treatment and are thus a potential human health hazard (7).

What is not known is the medical significance of small amounts of viral contamination which may occur in treated or potable waters. Current technology is capable of detection of small quantities of viruses in relatively large amounts of water. Decisions to require widespread application of this technology to water treatment plants have been discussed but have not been established, for both economical and scientific reasons. The establishment of standards for allowable virus content of treated waters is difficult with the current lack of knowledge on the proven health effect of low-level viral contamination of water supplies. We felt that demonstration of the critical level of contamination of water that produced a proven effect (infection, illness) in individuals would provide a basis for more intelligent decisions regarding the establishment of standards for viral content of treated waters. We also felt that, initially, experimental viral challenge studies whereby conditions of viral exposure, susceptibility of subjects, and intense observation and laboratory sampling could be rigidly controlled, could provide significant insight into this problem. Thus, we planned to conduct a series of studies which would define the minimal infectious dose of a representative of a viral group (ECHO viruses) frequently found in water, when that virus was ingested by susceptible subjects under relatively natural conditions (in drinking water).

Previous studies designed to determine minimal infectious doses of viruses involved artificial routes and vehicles of inoculation which might significantly affect the integrity of the virus and its effect (9). Specifically, virus was not "fed" in drinking water ingested under the usual conditions. In our study, we planned to suspend the viral challenge in the vehicle of concern, i.e., drinking water.

The specific goals of the study were to determine in susceptible, healthy, adult subjects who ingested water seeded with varying concentrations of ECHO-12 virus: (1) the minimal infectious dose, and (2) the medical significance of small amounts of an enteric virus ingested in this manner.

Our laboratory has had considerable experience in conducting viral challenges in human subjects. This experience is important to be able to achieve a successful study from the aspects of subject selection, compliance and cooperation, specimen collection, and accurate clinical evaluation. The use of artificial viral challenge in humans as means of studying various aspects of clinical virology (etiology, pathogenesis, prevention, therapy) have the advantages of rigidly controlling variables which are difficult to control under natural conditions: specific virus inoculation, viral dose, time of exposure, avoidance of exposure to other viruses, accessibility for examination and collection of specimens, known susceptibility status. And, specifically for the present study, we had previously conducted a viral challenge study using ECHO-12 virus so that a safety-tested pool was available, and data on the clinical and laboratory (virologic and serologic) sequelae to infection with the virus were known (10). The ECHO-12 virus was also desirable as the challenge material because it represented a group of viruses associated with contamination of treated waters and historically had not been associated with serious human illness (11-12).

The proposed study was to select healthy, young adult male subjects who lacked detectable serum hemagglutination-inhibition (HI) antibodies to ECHO-12 virus, place them in isolation, inoculate the subjects with varying doses of the virus or placebo (tissue culture fluid) and follow them for evidence of illness, viral shedding in the pharynx and intestinal tract, and antibody response. The assignment of inoculum (viral dose or placebo) was done by random selection, and all clinical and laboratory assays performed under double-blind, placebo-controlled conditions.

SECTION 2

CONCLUSIONS

This project represents the first tightly controlled investigation on minimal infectious doses of viruses in water in which non-attenuated challenge virus was utilized. The project consisted of a series of six human volunteer challenge studies conducted over a period of 2 years. The individual studies were conducted under similar conditions so that data derived from each study could justifiably be combined.

Based on a plaque assay utilizing LLC-MK_2 cells and ECHO-12 virus ingested in non-chlorinated drinking water, the following conclusions can be made:

1. No clinical illness occurred in doses up to 10,000 plaque forming units (pfu).

2. Infection was detected in 30% of the subjects given 10 pfu indicating that the minimum infectious dose may be considerably lower. Using statistical analysis and extrapolation from the experimental data, 1-2 pfu may be infectious for 1% of the population.

3. Intestinal shedding at doses as low as 10 pfu may persist for at least 19 days. At higher doses, the persistence may be longer.

4. Secondary spread was not detected among susceptible adult contacts.

Other conclusions that warrant further investigation may be stated as follows:

1. Virus ingested in drinking water may sometimes be recovered from the pharynx.

2. Consistent significant seroconversion probably requires a greater dose, or different route of inoculation.

3. Ingestion of a chlorine-inactivated, purified virus preparation at a concentration up to 100 pfu did not yield a detectable infectious or immune response.

SECTION 3

RECOMMENDATIONS

The current project has shown that human volunteer viral challenge studies can be used to provide important information on the role and medical significance of viruses in ingested water and to aid in the establishment of standards concerned with viral contamination of potable waters. In addition, the project has provoked interesting questions which should be considered for further investigations. Therefore, the following recommendations are made:

1. Projects of similar type should be conducted using different viral inocula that might have more medical significance (yet still be safe), e.g., other enteroviruses, rotaviruses.

2. The ECHO-12 virus work should be extended to include subjects with naturally acquired pre-existing serum antibody, and subjects from the current study who received virus and did develop antibodies as well as some who received virus but did not develop antibodies.

3. Consideration should be given to studying the role of multiple viral ingestion (i.e., more than one viral type at the same time; the same virus several times) and different routes of inoculation, e.g., aerosol.

4. Future studies should include the collection of pharyngeal washings rather than swabs to further investigate the pharyngeal shedding following viral ingestion in water.

5. Attempts should be made to define the viral particle content in relation to pfu in infective dose studies.

6. Evaluation should be made of newer cell lines for sensitivity to determine infectivity titers for challenge viruses. If more sensitive cell lines are found additional studies should be performed to directly measure viral infectivity at lower doses.

7. Viral shedding should be more accurately quantified to determine the relationship between dose and viral replication.

8. The relationship between viral infection and local (intestinal) antibody production should be determined, as well as the length of time such antibody is produced.

SECTION 4

RESEARCH DESIGN AND PROCEDURES

STUDY GOALS

This study has two main goals. These are to define the minimal infectious dose of an enteric virus ingested in water by susceptible, healthy subjects and to provide information to aid in the determination of the medical significance of relatively small amounts of an enteric virus ingested in water.

STUDY SEQUENCE

The original approach to the study was to administer two arbitrarily selected small doses of virus (10 and 100 pfu) or placebo to subjects and to further adjust the dosage based on this original study. It also was decided to "mimic" natural conditions as far as possible, so "tap" water was used as the vehicle for viral administration. Because tap water used in the original study had a high chlorine content, it was unsuitable for our purposes. Therefore, all subsequent studies were conducted with non-chlorinated, distilled, chilled Talawanda water.

A second but broader range-finding study was then performed in which small numbers of subjects received doses from 10 to 10,000 pfu. No placebo controls were included.

The remaining four studies focused on the "critical" dosage level suggested by study 2, and always included placebo controls.

STUDY PLAN

The six studies followed the same general plan. Subjects were selected based on lack of detectable serum antibody (at a 1:5 dilution) to the ECHO-12 challenge virus and evidence of good health. The subjects entered the isolation area on day 0 and remained for 8 days and 7 nights. Generally, subjects slept two to a room but freely intermingled. All meals were served as a group. No visitors were permitted. Virus or placebo was administered at 2 hr after breakfast on day 1. Following administration, only ingestion of distilled Talawanda water was permitted for 8 hr. No medications were permitted during the isolation period unless approved by study personnel. During the period of isolation, subjects were examined twice daily, and vital signs (temperature, pulse) were recorded at least twice daily. Pharyngeal and rectal swabs for viral isolation were collected at the time of the morning examination. After discharge from the

isolation area, subjects were seen serially (two or three times per week) for the next 3 weeks. Serial blood specimens were collected for serological analyses and for follow-up laboratory tests, i.e., complete blood count (CBC), SGOT, SGPT, and BUN. A summary of the study plan is presented in Table 1.

No subjects were used in more than one study; thus, no "cross-over" challenges were done. The parameters to be measured were clinical illness, viral shedding, and antibody response.

SUBJECTS

Subjects were selected primarily from area college students. Announcements pertaining to the study were posted in dormitories and college newspapers. Subjects were required to be in good health, age 18-45 years of age, and male. An initial blood specimen was collected which was tested for HI (hemagglutination inhibition) antibody. Subjects who lacked detectable HI antibody were recalled and given a general physical examination. Laboratory tests to assure good health status were also conducted. These included CBC, urinalysis, SGOT, SGPT, and BUN. If the subjects were considered in good health, written informed consent was obtained and the subject was permitted to participate in the study.

ISOLATION FACILITIES

Three sites were used for isolation of subjects: King's Island Inn, 20 mi north of Cincinnati; Sanders Hall, a dormitory on the main campus of the University of Cincinnati; and St. Edmund's Center in Glendale, Ohio.

King's Island Inn was used in four of the studies. In this location, subjects were housed in a separate wing, one or two to a room (and bath), permitted to intermingle freely, and ate meals in a common room. They also were permitted to use outside recreational facilities (fields, shuffleboard, basketball court), provided only persons participating in the study were present. No effort was made to segregate placebo or viral inoculated subjects in terms of room sharing. The studies done at King's Island were conducted during the "off" season when guest numbers were low (winter, and early spring). Sanders Hall, a dormitory on the main campus of the University of Cincinnati, was used for one study. A suite consisting of a large day room, 10 bedrooms, and two bathrooms was used. Subjects slept one or two to a bedroom, intermingled freely during the day, and ate in the common area. They were restricted to these facilities. St. Edmund's Center in Glendale, Ohio, was used for one study. The facilities (a single building) were dormitory-like with two to five persons sleeping in the same room, and three common bathrooms. A common eating area was available within the building, and all food was prepared in the kitchen. The building was surrounded by large playfields, which the subjects were permitted to use.

CHALLENGE VIRUS

The challenge virus used in the study was ECHO-12 virus. This virus was originally recovered from a child with a clinical diagnosis of erythema

infectiosum (fifth disease). The isolate was identified by Dr. Edwin Lennette in the California State Laboratories. A pool had been produced and safety-tested according to acceptable standards (13). The virus had been passaged twice in monkey kidney tissue culture, and the final cell-free harvest had a titer of approximately 1×10^7/ml based on direct cytopathic effect. This pool had been administered intranasally to susceptible (lacking serum HI antibody to ECHO-12) subjects in a study designed to demonstrate the role of ECHO-12 in the etiology of erythema infectiosum and to determine the effect of viral infection on cell mediated immune mechanisms. Up to 10^6 infectious viruses were administered intranasally without the production of serious illness (10). The subjects developed mild to moderate headache and low-grade fever, shed virus for 1 to 4 days, and experienced a significant HI antibody response.

The safety-tested stock of ECHO-12 virus used in the present study was clarified by differential centrifugation and further purified by sucrose gradient centrifugation. Viral fractions from the gradient (15-30% w/v) were detected by absorbance at 254 nm. Peak fractions were pooled, diluted 10-fold in phosphate buffered saline (PBS) and stored at -70°C. The pre-inoculation titer of the pool was 1.6×10^6 pfu /ml as determined in LLC-MK_2 cells, a rhesus monkey kidney cell line.

ADMINISTRATION OF THE VIRUS

Before administration to volunteers, a sufficient quantity of viral suspension was prepared by dilution of the stock ECHO-12 pool into chilled PBS and kept on ice. The aliquots to be given to the subjects were prepared, just before administration, by the addition of 1 ml of viral suspension to each individual cup containing 100 ml of chilled drinking water. Cups were all color-coded by viral content and each subject received and immediately consumed the contents of 1 cup. The sequence of administration of different viral doses was pre-determined by computerized, random selection. Aliquots of each viral suspension were kept chilled and titered within 24 hr by the plaque assay.

PLAQUE ASSAY OF ADMINISTERED ECHO-12 VIRUS

One ml of the virus suspension was added to each of about fifty 75-cm^2 flasks containing a confluent monolayer of LLC-MK_2 cells grown in Eagles MEM with 10% fetal calf serum and antibiotics. Five flasks were inoculated with 1 ml each of Talawanda water as controls. After 2 hr of adsorption time at 37°C, 20 ml of pre-warmed 1% agar overlay medium was added to each flask. The medium contained 1% agar, in Hank's balanced salt solution, 3% fetal calf serum, and antibiotics. Once the agar had solidified, the flasks were inverted. After 5 days incubation, a second overlay was added with neutral red. Exposure to light was avoided. Appearance of plaques was observed after 24 hr.

VIRAL ISOLATION AND IDENTIFICATION

Rectal and pharyngeal swabs were rinsed into 2-2.5 ml of PBS and 0.3 ml was inoculated into each of two culture tubes of LLC-MK_2 cells on a

maintenance medium of Eagles MEM with 2% fetal calf serum. The tubes were incubated in a stationary position at 37°C. All tubes underwent medium change twice weekly and were inspected daily for cytopathic effect (CPE) for 14 days. Positive isolates were harvested and frozen at -70°C. Those cells showing no CPE after 14 days were subcultured into fresh LLC-MK_2 cells and followed for 7 days. Positive isolates were identified by neutralization assay using standard ECHO-12 antiserum.

ANALYSES OF ANTIVIRAL ANTIBODIES

Two tests were used during this study to detect humoral antibodies against ECHO-12.

HI Antibody Assays

Standardized viral HI antibody tests were performed (15). Briefly, the sera were treated (kaolin adsorption) by mixing a 1:5 dilution of serum in dextrose gelatin veronal buffer (DGV) for the removal of nonspecific inhibitors. After a 20 min incubation at room temperature, the mixture was centrifuged briefly to sediment the kaolin. The supernatant fluid was then adsorbed with 0.1 ml of a 50% suspension of human O erythrocytes. After 1 hr at 4°C, the samples were centrifuged for 15 min at 500 x g and the supernatant fluid was used for the conventional HI antibody test.

Microtiter Neutralization Test for ECHO-12 Antibody

The tests were conducted in disposable microtiter "U" plates. Pre-sera and post-sera were inactivated at 56°C for 30 min. Twofold dilutions of each serum were prepared in Eagle's MEM medium containing 2% fetal calf serum (FCS) using 25 µl diluting loops. To each serum dilution was added 25 µl of ECHO-12 virus containing 100 $TCID_{50}$ (as determined by previous titration). The serum-virus mixtures were incubated at room temperature for 1 hr. A suspension of 10^4 LLC-MK_2 cells in 50 µl MEM + 5% FCS was added to each well on the microtiter plate. The wells were read for CPE after 4 days of incubation.

CLINICAL EVALUATION

Subjects were examined twice daily (A.M. and P.M.) during the period of isolation and at the time of follow-up outpatient visits. During these examinations, signs and symptoms were scored according to the following scale: absent = 0, mild = 1, moderate = 2, severe = 3. In addition, twice-daily vital signs were recorded. The person conducting the clinical evaluation was not present at the time of administration of inocula, so that double-blind conditions would be maintained. A summary of the clinical evaluation is presented in Table 2.

SECTION 5

RESULTS

SELECTION OF SUBJECTS FOR STUDY

Applicants for participation in the study numbered 474. Of these, 417 (88%) lacked pre-existing serum HI antibodies at a 1:5 dilution level. All susceptible applicants were recalled for a general physical examination and about 15% of those who returned were disqualified on a medical basis. All persons who qualified were asked to participate in one of the studies. However, because of the spread of time between the initial screening and the later studies, many of those qualified were no longer interested by the time they were scheduled to report. The actual number of participants was 189. A summary of program participants is presented in Table 3.

SUMMARY OF STUDIES

Six separate studies were carried out in which volunteers were given between 0 and 10,000 pfu, as determined in LLC-MK_2 cells of safety-tested ECHO-12 virus in chilled drinking water. The studies were performed over a period of nearly 2 years at three different locations. A summary of the number of participants, dosage administered, location and time of the study is presented in Table 4.

Study 1 involved 40 subjects. Thirty of these received a virus inoculum seeded into 100 ml of chlorinated tap water at the isolation site. We later performed laboratory assays in which virus was inoculated into the tap water, having a free residual chlorine of 0.8 mg/l, and showed no survival of infectious virus by 45 minutes, a period approximately equal to time of virus suspension in tap water before ingestion by the subjects. While it was highly unlikely that the subjects received a viable viral challenge because of the presence of chlorine in the tap water, laboratory assays were conducted to see if any infectious ECHO-12 virus could be detected in pharyngeal or rectal swabs of subjects taken during the week of isolation. In addition, sera samples taken 26 days after viral inoculation were tested for antibody to ECHO-12. Data from subsequent studies indicate that 18 of the subjects dosed in this experiment would likely experience infection if the inocula were fully viable. However, there was no illness or evidence of infection in any subject that ingested the seed virus suspended in chlorinated tap water.

Study 2 was a "range-finding" study and did not involve any placebo subjects. The results are summarized in Table 5. These results indicated that subsequent studies should concentrate on dosages between 10 and 100

pfu and optimally should include dosages of less than 10 pfu. Attention was directed toward construction of a "dose-response" curve from which extrapolation to lower dosage could justifiably be made. Studies 3-6 were designed to provide data which could be used to establish the shape of this curve.

DETAILED ANALYSIS OF STUDIES

Placebo Subjects

None of the 34 placebo subjects who participated in Studies 3 through 6 experienced significant illness or evidence of infection (viral shedding and/or significant seroconversion). The placebo subjects intermingled freely with viral-dosed subjects, yet no evidence of secondary (contact) infection was found. The lack of contact spread of infection probably reflected the age of the subjects, the personal hygiene stressed by the study personnel, and the cleanliness of the isolation facilities.

Viral Isolation

Attempts were made to detect evidence of viral replication in subjects through analysis of pharyngeal and rectal swabs. These were collected daily while the subjects were in isolation and in all follow-up visits during the 3-week period after isolation. ECHO-12 was the only virus recovered by either method.

Pharyngeal Swabs--

Recovery of ECHO-12 virus from pharyngeal swab specimens is summarized in Table 6. Approximately 7100 specimens were anlyzed. ECHO-12 virus was recovered from the pharynx of 8 of 137 subjects tested. Of those that received virus (103), pharyngeal shedding was detected in less than 8%. Virus was found in the pharynx of six subjects receiving 10 pfu but virus was detected in the stool of only one of these six. Furthermore, there was no evidence of "dose-response" relationships in terms of detectable pharyngeal shedding. Finally, the virus could be recovered only on one day from the positive subjects, and most of these isolates required subpassage before they could be detected. Therefore, the significance of pharyngeal viral shedding was unclear, and was not used to determine incidence of infection.

Rectal Swabs--

Table 7 summarizes the incidence of recovery of ECHO-12 from the rectal specimens. Approximately 7300 specimens were tested. Based on percentages of intestinal viral shedders found, a definite "dose-response" relationship was established.

The patterns of intestinal viral shedding are depicted in Figures 1 through 4. Two features were evident. First, the bulk of the viral shedding occurred during the first week after inoculation regardless of inoculum dose. Second, although the lower dose of 10 pfu appeared to result in lower concentration of virus recovered in the first week (as shown by the more frequent requirement for subpassage for detection), the

length of time viral shedding occurred did not appear to be dose dependent. It should be noted that rectal swabs were collected and analyzed for all the days specified in Table 1 for every subject, but only the positive results are shown in these Figures.

Serology

Two assays were used to measure increases in humoral antibody against ECHO-12 virus: microtiter neutralization (MN) and hemagglutination inhibition (HI). Both assays detected nearly the same number of subjects with increased antibody to ECHO-12. Nine subjects showed increases by both techniques and the increase detected was significantly greater when measured by MN than by HI in eight. The same increase was detected by both methods in the other subject. Most increases were detected by only one of the two techniques. Twelve were detected only by HI and thirteen were seen only by MN. Large increases in antibody titer were rare. Only ten subjects displayed increases of 8-fold or greater by either technique and only five of these were 32-fold or greater. Table 8 summarizes the serological data and presents it in relation to the intestinal shedding results. The total percentages of subjects positive for virus replication by either intestinal shedding or humoral antibody response in each dosage group is also presented.

Statistical Analysis

The dose response relationship between virus dose and infectivity was estimated using probit analysis (16). For this analysis, infectivity was defined as a significant seroconversion and/or evidence of intestinal viral shedding. The results from four dose levels (10, 30, 100, and 300 pfu) were included in the analysis (Table 8). The probit transformation was used because it is based on a normal tolerance distribution and the assumption of normality seems the most appropriate for this type of data, i.e., when it is probably that differences in individual susceptibility are the primary reasons for differences in response among subjects in the same dose groups. A Chi-square goodness-of-fit test indicated that there was no significant departure from the assumed relationship and the observed data (p 0.39).

Based on the estimated response curve, the median infectious dose (ID_{50}), i.e., the dose that would on the average produce a response in 50% of the subjects, is 28 pfu of ECHO-12 virus with 95% fiducial limits of (17,45) pfu. The estimated ID_{01} is 1 pfu with 95% fiducial limits of (0.03,1.7) pfu. These results are depicted in Figure 5.

Caution should be taken in the inference drawn from the ID_{01} estimate. In this study, estimation of the ID_{50} is much more reliable than estimation of the ID_{01}. The precision of the estimates depends on the doses tested and extrapolation beyond the range of observations can be misleading. Ideally, the ID_{01} should be estimated from observations made around its dosage level. In this study, this was not possible. It was determined that a dose of 10 pfu was the lowest dose that could be administered with any degree of reliability. Since the study aim was to determine

a minimal infectious dose, more subjects were tested at the lowest possible dose. Another problem in estimating the minimal infectious dose is the choice of the transformation for the response curve. Different transformations would likely result in different estimates. Response curves must be estimated from the data and the regression line may not be the same in the region of the ID_{01} as in the region of the ID_{50}. Deviations from the assumed association are likely to be more important for extreme doses.

Clinical Summary

None of the subjects in the entire program developed any significant illness. Two subjects who received 100 and 10,000 pfu, respectively, reported "diarrhea" for 24-36 hr, although this was more accurately defined as "soft formed stools." A third subject who received 100 pfu developed a "rash" which appeared to be a 24-hr "heat" rash. No abnormalities were found by the hematological and chemical tests and urinalyses as a result of the viral challenge at any of the dosages used.

SECTION 6

DISCUSSION

The results of this study indicate that an intestinal infection with an enteric virus can be initiated with a very low concentration of infectious viruses consumed in drinking water. The final statistical analysis of the results suggests that this concentration may be as low as one in 1 out of 100 susceptible, healthy adults. If this is generally true of enteric viruses, their presence in low concentration in drinking water poses a significant risk to human health. It also indicates that drinking water containing infectious viruses could serve as a vector in the spread of enteric viral diseases.

Because of the implications of the conclusions reached in this study with regard to public health policies and practices, the data on which these conclusions were reached must be examined in detail. In the first place, the virus used in this study was chosen because it had been used in previous studies and was found to be "safe." However, it caused no disease and, therefore, is only a model for enteric viruses that do cause disease. Similar experiments with one of these agents are needed to test the conclusions drawn from this study with ECHO-12 virus.

The method used to determine the number of infectious viruses given to volunteers in this study was the plaque assay, using a continuous line of monkey kidney cells. Although the values obtained by this accepted method were quite reproducible, some assays and cell lines will be more sensitive and others less sensitive. This point must always be taken into consideration when virus numbers are accurately quantified. If a less sensitive method had been used, the minimum infectious dose determined in this study would have been lower; if a more sensitive method were used, the minimum dose would have been higher.

A number of features of this study suggest that the minimum infectious dose detected may be even lower than that found, i.e., a larger percentage of certain persons may replicate virus when infected with just one pfu. The study subjects were healthy adults who had no detectable humoral antibody to ECHO-12 virus at a 1:5 dilution of sera as determined by an HI test. Other persons (immunosuppressed adults, infants) are likely to be much more susceptible. In addition, the adult subjects studied may have had a low but undetected level of humoral, neutralizing antibody or may have had intestinal antibody to ECHO-12. Finally, consumption of the viruses with food or liquids other than water (as used in this study) may better protect these viruses during passage from mouth to intestine. These factors should also be taken into consideration.

It is interesting that many persons found shedding virus in this study had little or no detectable increase in humoral antibody to ECHO-12 virus 26 days after infection. This could have been due to the low viral numbers used for infection or, possibly, the route of infection. The original study done with ECHO-12 virus in the early 1970's revealed seroconversions in most susceptible persons but here the virus was given intranasally at a relatively high dose (10^6 $TCID_{50}$). Although 26 days is expected to permit sufficient time to "seroconvert" following infection, humoral antibody titers may have risen significantly after this period. If this were the case, the calculated minimum infectious dose could have been lower.

If these points are all taken into consideration, it should be possible to state with some certainty the minimum infectious dose of ECHO-12 virus taken orally and measured by the methods described here. Statistical analysis of the results indicates that this minimum dose is no larger than one for infection of about 1% of a healthy adult population lacking antibody to the virus. The analysis used makes assumptions that cannot be proven without additional data because it is based on a statistical extrapolation of results where 10 or more pfu were administered to subjects. If more sensitive methods could be found to detect infectious viruses, additional studies could be performed to directly measure viral infectivity at lower doses than those used in this study.

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TABLE 1. SUMMARY OF STUDY PLAN

	DAY	ADMINISTRATION OF VIRUS	SYMPTOMS RECORDED	PS & RS TAKEN	SEROLOGY
ISOLATION	0	X	X	X	X
	1				
	2-6		X	X	X
	7		X	X	
	9		X	X	
	12		X	X	
	16		X	X	X
	19		X	X	
	23		X	X	
	26		X	X	X

TABLE 2. CLINICAL FEATURES
EVALUATED AND SCORED

HEADACHE SORE THROAT DIARRHEA NAUSEA VOMITING RASH MALAISE URI ADENOPATHY OTHER	ABSENT = 0 MILD = 1 MODERATE = 2 SEVERE = 3

FEVER: 99°F = 0
99.2-100° = 1
100.2-101° = 2
101.2° = 3

TABLE 3. SUMMARY OF PROGRAM PARTICIPANTS

Number who volunteered:	474
Number susceptible*:	417 (88%)
Number returned for physical exam:	∿350 (∿80%)
Number qualified following phys. exam:	∿300 (∿75%)
Number participants in full program:	189 (40%)

*Lacked detectable hemagglutination-inhibition (HI) serum antibody.

TABLE 4. SUBJECTS AND DOSAGE ADMINISTERED IN EACH VIRAL CHALLENGE STUDY

Study No.	Total No. subjects	Dosage administered		Location*	Date
		Dose (pfu)	No. subjects		
1**	40	p***	10	KII	Mar. 1979
		10	10		
		100	20		
2	17	10	5	SH	June
		100	5		
		1,000	4		
		10,000	3		
3	32	P	11	KII	Dec. 1979
		100	21		
4	47	P	15	KII	Mar. 1980
		10	32		
5	10	P	3	St.E	June 1980
		30	7		
6	43	P	5	KII	Dec. 1980
		10	13		
		30	13		
		300	12		

*Location: KII = Kings Island Inn; SH = Sanders Hall; St.E. = St. Edmonds Conference Center

**Chlorinated water used as vehicle.

***P = placebo (control).

TABLE 5. SUMMARY OF RANGE-FINDING STUDY (EXPERIMENT 2)

No. of subjects	Dose ECHO-12 virus administered (pfu)	Viral shedding only	Seroconversion only*	Viral shedding + seroconversion	Total No. subjects infected
5	10	4†	0	0	4
5	100	4	0	2	4
4	1,000	2	0	2	2
3	10,000	2	0	1	2

*Seroconversion = 4-fold or greater rise in antibody by either microtiter neutralization or hamagglutination inhibition.

†One subject, virus was recovered from pharyngeal swab only; one subject, viruses were recovered from both rectal and pharyngeal swabs.

TABLE 6. RECOVERY OF ECHO-12 VIRUS FROM PHARYNGEAL SWAB (PS) SPECIMENS

Dose (pfu)	No. subjects with positive PS/ no. subjects tested	Percent positive
0	0/34	0
10	6/50	12
30	0/20	0
100	1/26	4
300	ND*	ND
1,000	1/4	25
10,000	0/3	0

*ND = not done.

TABLE 7. INCIDENCE OF INFECTION BASED ON RECOVERY OF ECHO-12 VIRUS FROM RECTAL SWABS (STUDIES 2-6)

Dose (pfu)	No. subjects viral positive/ no. challenged	Percent positive
0	0/34	0
10	14/50	28
30	8/20	40
100	18/26	69
300	11/12	92
1,000	2/4	50
10,000	2/3	67

TABLE 8. CORRELATION OF INTESTINAL SHEDDING OF ECHO-12 VIRUS AND SEROCONVERSION (STUDIES 2-6)

Dose (pfu)	No. subjects	No. subjects positive for intestinal shedding of virus	No. subjects with equivocal seroconversion[a]	No. subjects with significant seroconversion[b]	Total subjects positive by either method[c]
0	34	0	0	0	0/34 (0%)
10[d]	50	14	2	7	15/50 (30%)
30[d]	20	8	5	3	9/20 (45%)
100[d]	26	18	1	11	19/26 (73%)
300[d]	12	11	0	3	12/12 (100%)
1,000	4	2	1	1	2/4 (50%)
10,000	3	2	0	1	2/3 (67%)

[a]Equivocal seroconversion = HI antibody level increased from <1/5 to 1/5, or neutralization antibody increased from < 1/2 to 1/2.

[b]Significant seroconversion = fourfold increase or greater in HI antibody and/or neutralization antibody.

[c]Only subjects with significant seroconversion were included; 2 subjects (one at 10 pfu and one at 30 pfu) that were not found to shed virus but had equivocal seroconversion are not included.

[d]One subject developed a significant serological response without evidence of viral shedding at each of these doses.

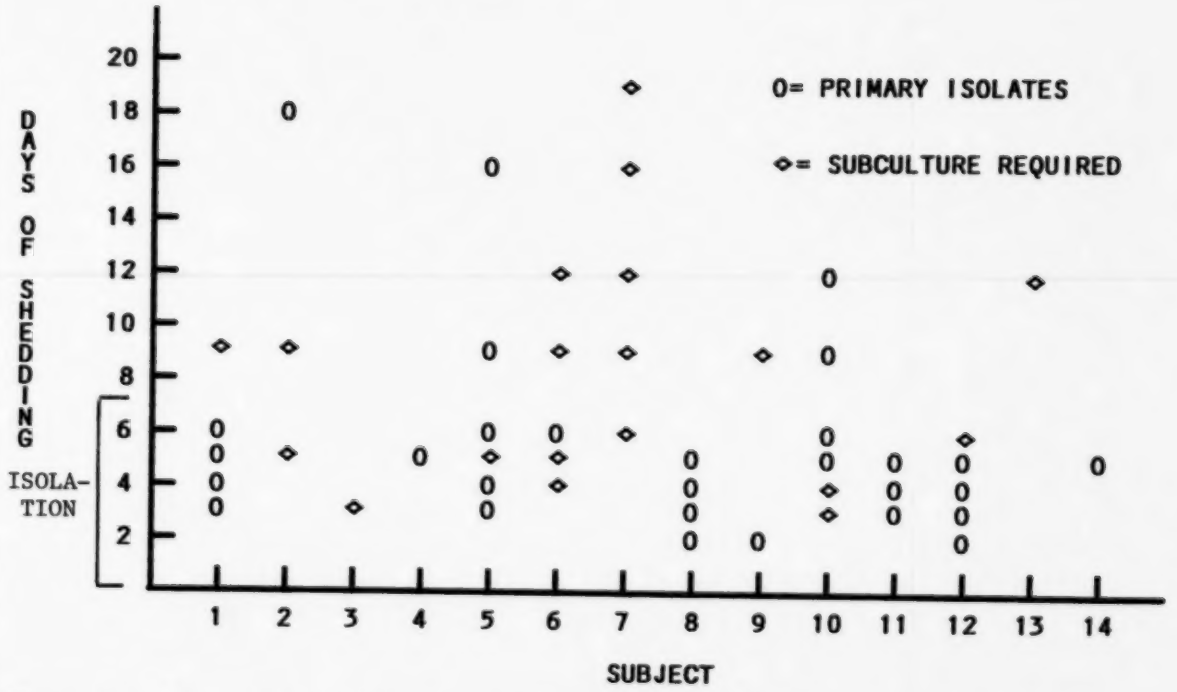

Figure 1. Intestinal shedding patterns among the subjects who received 10 pfu ECHO-12 virus (positive subjects only).

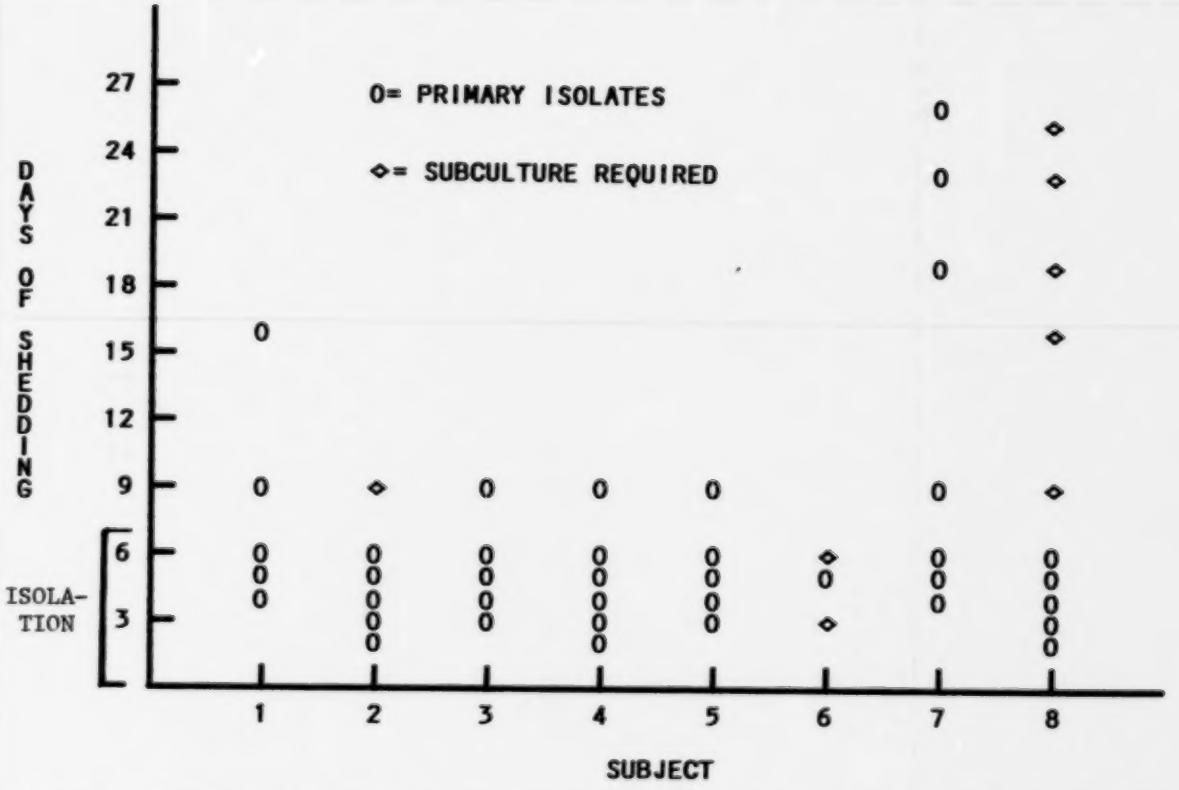

Figure 2. Intestinal shedding patterns among the subjects who received 30 pfu ECHO-12 virus (positive subjects only).

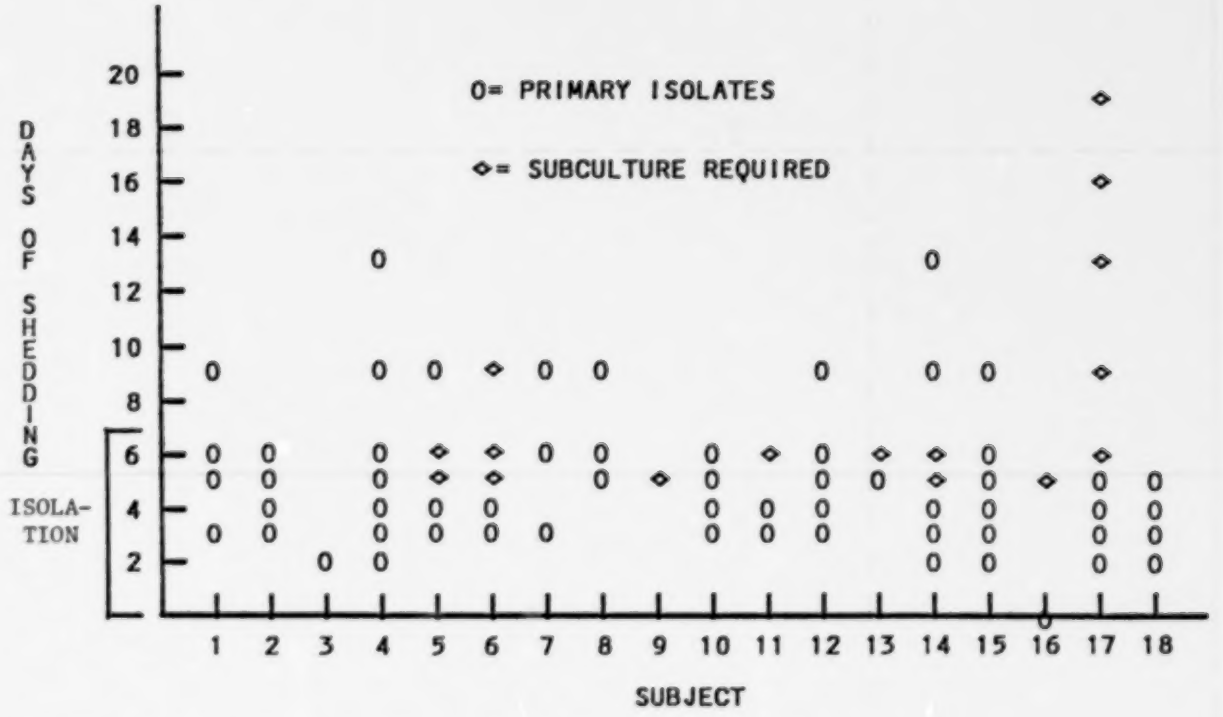

Figure 3. Intestinal shedding patterns among the subjects who received 100 pfu ECHO-12 virus (positive subjects only).

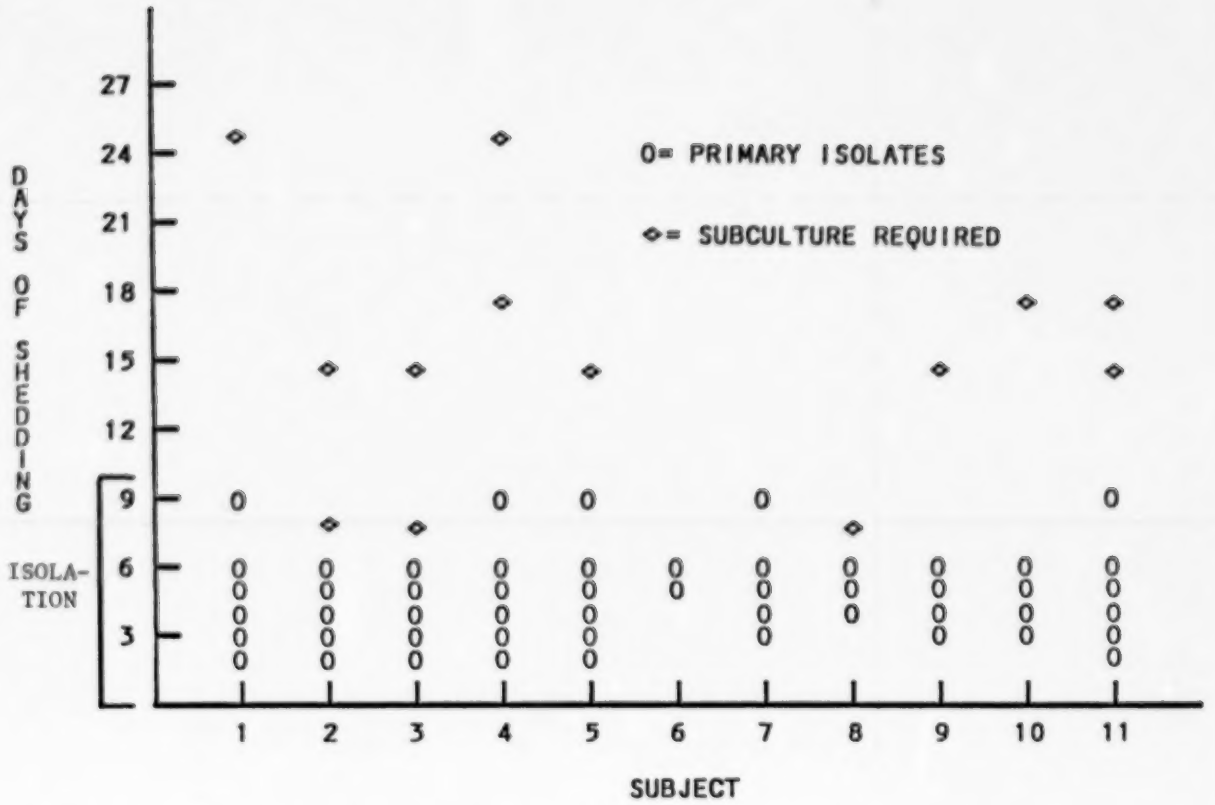

Figure 4. Intestinal shedding patterns among the subjects who received 300 pfu ECHO-12 virus (positive subjects only).

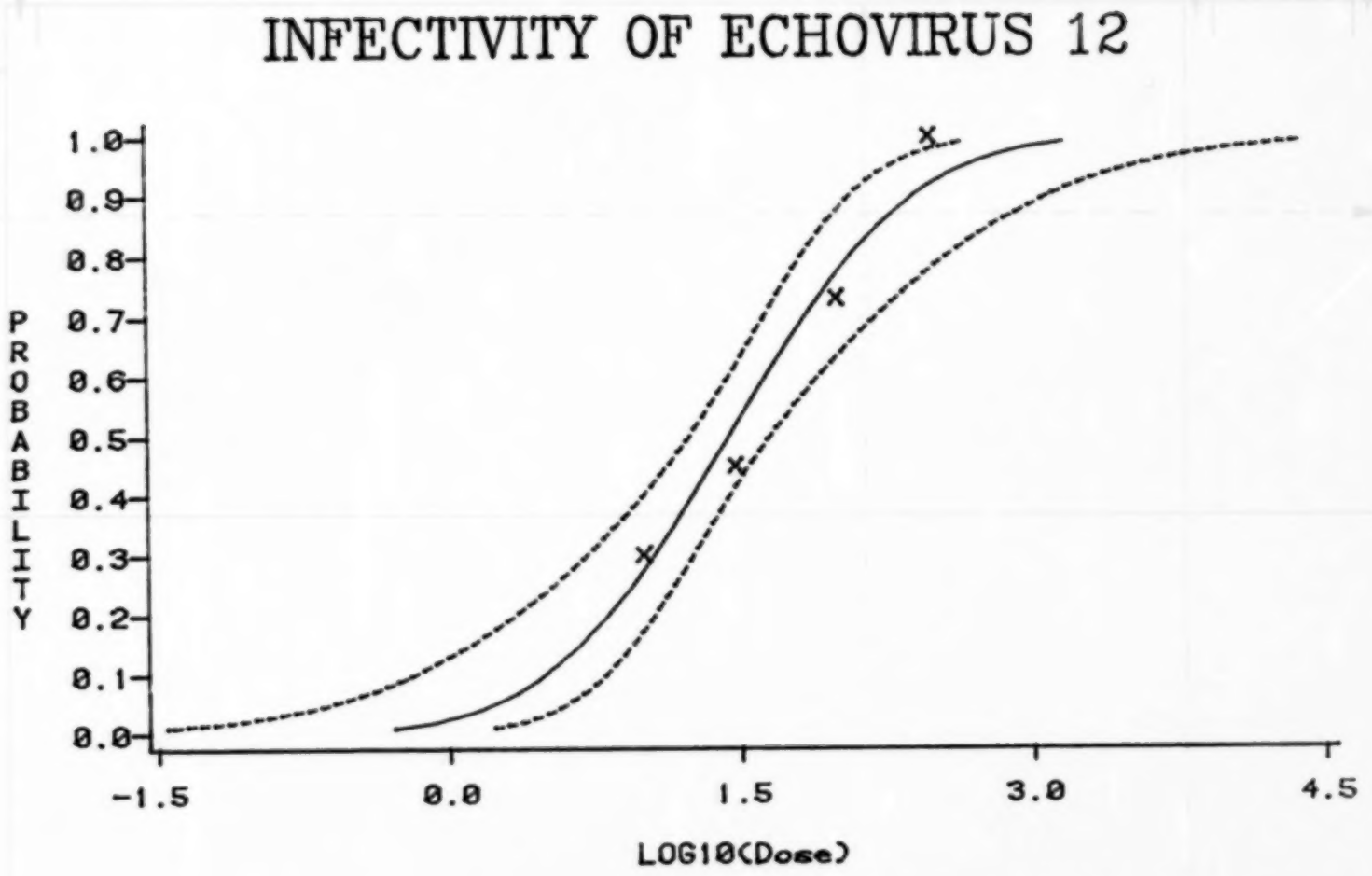

Figure 5. Probit analysis of the probability of infection with 95% fiducial limits.